Cellular Cardiomyoplasty Improves Survival After Myocardial Injury

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Background—Cellular cardiomyoplasty is discussed as an alternative therapeutic approach to heart failure. To date, however, the functional characteristics of the transplanted cells, their contribution to heart function, and most importantly, the potential therapeutic benefit of this treatment remain unclear.

Methods and Results—Murine ventricular cardiomyocytes (E12.5–E15.5) labeled with enhanced green fluorescent protein (EGFP) were transplanted into the cryoinjured left ventricular walls of 2-month-old male mice. Ultrastructural analysis of the cryoinfarction showed a complete loss of cardiomyocytes within 2 days and fibrotic healing within 7 days after injury. Two weeks after operation, EGFP-positive cardiomyocytes were engrafted throughout the wall of the lesioned myocardium. Morphological studies showed differentiation and formation of intercellular contacts. Furthermore, electrophysiological experiments on isolated EGFP-positive cardiomyocytes showed time-dependent differentiation with postnatal ventricular action potentials and intact $\beta$-adrenergic modulation. These findings were corroborated by Western blotting, in which accelerated differentiation of the transplanted cells was detected on the basis of a switch in troponin I isoforms. When contractility was tested in muscle strips and heart function was assessed by use of echocardiography, a significant improvement of force generation and heart function was seen. These findings were supported by a clear improvement of survival of mice in the cardiomyoplasty group when a large group of animals was analyzed (n=153).

Conclusions—Transplanted embryonic cardiomyocytes engraft and display accelerated differentiation and intact cellular excitability. The present study demonstrates, as a proof of principle, that cellular cardiomyoplasty improves heart function and increases survival on myocardial injury. (Circulation. 2002;105:2435-2441.)

Key Words: transplantation ■ cells ■ electrophysiology ■ contractility ■ survival

Cardiovascular diseases are the most frequent cause of death in the western hemisphere. The critical loss of functional cardiomyocytes causes a severe deterioration of pump function, resulting in heart failure. Because differentiated cardiomyocytes lack prominent regenerative capacity, heart transplantation remains the only effective causal therapy. Because of the increasing number of patients requiring this treatment and the decline in available donor organs, alternative methods, such as cellular cardiomyoplasty,1−3 are urgently needed. Thus far, however, convincing evidence showing a clear therapeutic benefit of this approach is lacking.

To compare survival in a large group of mice after heart injury alone or combined with cellular cardiomyoplasty, we used an operative procedure with very low mortality.4 A combination of morphological, functional, and molecular methods enabled us to gain detailed insight into differentiation, physiological function, and contractility of transplanted cells and their role for heart function.

Methods

The Animal Care Committee of the University of Cologne approved all the procedures performed on animals.

Mouse Breeding and Harvesting of Embryonic Cardiomyocytes

Transgenic mice5 of the strain HIM:OF1 or C57/Bl6 were bred and enhanced green fluorescent protein (EGFP)-positive embry-
onic ventricular cardiomyocytes (E12.5-E15.5) harvested as reported. After dissociation, the cells were resuspended in DMEM (20 000 cells/µL). Flow cytometry was performed as described.

**Operation and Cell Injection**

Male wild-type mice of the respective strains were used as recipients. The surgical procedure and the injection of cells (100 000 cells diluted in 5 µL of solution) were performed as reported. For control, EGFP-positive cardiomyocytes or solution without cells was injected into the intact myocardium or the cryolesioned myocardium, respectively.

**Histology, Immunohistochemistry, and Western Blot Analysis**

Morphological preparation and staining of tissue samples were performed as described. Nuclei were stained with DAPI (Vector Laboratories). To evaluate cross-striation, anti-α-sarcomeric actin (1:800, Sigma) primary and Cy3 goat anti-mouse (1:1000, Dunn Labortechnik), which recognizes both α1 isoforms. Comparable protein loading was assessed with Coomassie staining.

**Electrophysiology**

Single cardiomyocytes were isolated with Langendorff perfusion at 5 or 6 (early) and 11 to 14 (late) days after operation. Transplanted cardiomyocytes were identified on the basis of their EGFP expression. APs in cells of their slack length, and maximum Ca²⁺-activated force was determined within the injured area (data not shown). After 6 days, distinct fibrotic scar tissue formed and de novo synthesis of stable extracellular matrix became visible (bottom). This suggests that the most mechanically unstable period occurs after the necrotic degeneration of cardiomyocytes and before the fibrotic wound healing, ie, 2 to 6 days after cryoinjury.

Because of the differences in survival observed at early stages after operation (see below), our analysis focused on the first 2 weeks after cardiomyoplasty. During the first few days after operation, the transplanted cells were round and scattered within the injured area (data not shown). After 6 days, elongation and physiological alignment of cells took place. After 2 weeks, the implanted cardiomyocytes displayed transmural engraftment in the cryolesioned area (Figure 2a). The majority of transplanted cardiomyocytes were oriented in a manner similar to that of the native muscle fibers. In fact, ventricular-like rod-shaped cardiomyocytes with end-to-side and end-to-end contacts were found (Figure 2b). The cells were mononucleated, and the EGFP expression was restricted to cardiomyocytes (Figure 2c). Differentiation was also confirmed by distinct cross-striation detected with cardiac α-actin staining (Figure 2d) and demonstration of an intact myofibrillar apparatus by electron microscopy (Figure 2e, top right). The ultrastructural analysis revealed a large amount of rough endoplasmic reticulum in the transplanted cells, indicating the metabolically activated status of the cells (Figure 2e, bottom).
Within a few days after transplantation, intercalated disks were detected, suggesting the establishment of electromechanical coupling (Figure 2e, bottom right). The reduced size of the transplanted cardiomyocytes and the relatively thickened cross-striation suggest that the cells late after operation are not yet terminally differentiated. Taken together, our morphological studies demonstrate that the cryolesion is replaced by fibrotic scar tissue within a week.

Figure 1. Transgenic mouse and cryoinjury model. a, Transgenic embryonic (E13.5) heart displaying prominent enhanced green fluorescent protein (EGFP) expression under fluorescent light. The ventricles (V) and atria (A) can be recognized. b, Flow cytometry of freshly isolated transgenic ventricle-derived cells (E13.5). A large fraction of intact (propidiumiodide-negative) cardiomyocytes (EGFP positive cells) is detectable. Van Gieson staining (c) and (d) electron microscopy of changes occurring after cryoinjury. Upper, middle, and lower panels depict heart tissue 2, 4, and 6 days after lesion. In van Gieson stainings muscle tissue is yellow and fibrotic tissue is red (c). Two days after injury, myocytolysis with myofibrillar degeneration and mitochondrial damage is seen. From day 4 invasion of fibroblasts and de novo synthesis of extracellular matrix takes place (d). Bar=100 μm in c and 6 μm in d.

Figure 2. Morphological characterization of transplanted cardiomyocytes. a, Cross section of left ventricular wall shows engraftment of large numbers of EGFP-positive cardiomyocytes 2 weeks after operation. Note reduced wall thickness of infarcted area. Yellow color is a result of background fluorescence of infarcted heart muscle. b, Higher magnification evidences rod shape and end-to-end and end-to-side contacts (arrowheads) between transplanted cardiomyocytes. c, Costaining with DAPI shows that EGFP-positive cells are mononucleated. d, α-Actinin-stained transplanted cardiomyocytes display cross-striation; EGFP expression (inset) proves their transgenic nature. e, Ultrastructural analysis of transplanted cardiomyocytes in central area of cryoinjury. Two cardiomyocytes form side-to-side contacts (left). High magnification of transplanted cardiomyocyte depicted on left shows intact sarcomeric organization (top right). Intercalated disks can be detected (bottom right). Bar=110 μm in a, 20 μm in b and c, 15 μm in d, 45 μm in d (inset), 4 μm in e (left), and 2 μm in e (right).
Cellular cardiomyoplasty yields successful engraftment, physiological orientation, and differentiation of the implanted cells.

Functional Characteristics of Transplanted Cells

Next, we wondered whether the physiological characteristics of transplanted cells were similar to those of native cardiomyocytes. Because of their key role for heart function, we examined the expression of ion channels. Isolated transplanted cardiomyocytes could be easily identified because of their EGFP expression (Figure 3a) and functionally characterized with the patch-clamp technique. Early after operation, many round cells were observed, whereas late after operation, higher numbers of rod-shaped transplanted cells were seen. The time-dependent differentiation of transplanted cardiomyocytes was illustrated by comparison of the 90% AP duration ($\text{APD}_{90}$) of embryonic and transplanted cardiomyocytes. Early after operation, the transplanted cells displayed spontaneous electrical activity with depolarized resting membrane potentials ($56.3 \pm 0.3 \text{ mV}$, $n=3$) and prolonged $\text{APD}_{90}$ (208.3 $\pm$ 7 ms, $n=3$) (Figure 3c and 3e). The significant shortening of the $\text{APD}_{90}$ in early-postoperation cells compared with E12.5/E13.5 embryonic cardiomyocytes ($359.2 \pm 40 \text{ ms}$, $n=5$, Figure 3e) suggested differentiation. In fact, these early-postoperation cells resembled ventricular cardiomyocytes of the late embryonic/perinatal stage. At this stage, intact $\beta$-adrenoeceptor-mediated modulation, a hallmark for physiological heart function, was already seen, because isoprenaline (1 $\mu$mol/L) led to prolongation of the APD and a more positive plateau phase (Figure 3c). When late-transplanted cells were measured, stable resting membrane potentials ($-68 \pm 8 \text{ mV}$, $n=8$) similar to those of native cells ($-71.4 \pm 0.5 \text{ mV}$, $n=12$) isolated from the same hearts were found. In control (Figure 3b) and transplanted (Figure 3d) cells, electrical stimulation evoked the typical spiky APs with short-lasting $\text{APD}_{90}$s of 35.8 $\pm$ 0.8 (n=12) and 41.9 $\pm$ 2.4 (n=8) ms, respectively (Figure 3e). $I_{\text{Kf}}$ is critical for setting the membrane potential; therefore, current densities were determined. These were similar in wild-type (4.8 $\pm$ 0.4 pA/pF, n=10) and late-transplanted (4.5 $\pm$ 0.1 pA/pF, n=9) but significantly lower in early-transplanted (1.3 $\pm$ 0.2 pA/pF, n=5) cardiomyocytes. In early- and late-postoperation cardiomyocytes, the L-type $\text{Ca}^{2+}$ current, a key component of excitation-contraction coupling, displayed normal biophysical characteristics (Figure 3f) and similar current densities (Figure 3g, top). In line with the establishment of intact $\text{Ca}^{2+}$-induced$\text{Ca}^{2+}$ release during differentiation, the inactivation kinetics of the L-type $\text{Ca}^{2+}$ current were found to be accelerated in late-transplanted cells (Figure 3g, bottom). Occasionally, a tight connection between EGFP-positive and native cardiomyocytes was seen after the isolation. When lucifer yellow (3 mg/mL) was included in the patch pipette, the passage of dye from the patched EGFP-positive cell to the tightly connected native cardiomyocyte was observed (n=3), suggesting the expression of functional gap junctions between these cells. To gain more insight into the differentiation of transplanted cells, the expression pattern of TnI, known to switch at the perinatal stage from the slow skeletal to the cardiac isoform$^{13}$ (Figure 4a), was analyzed by Western

![Figure 3](http://circ.ahajournals.org/)
blotting. No TnI was detected in untreated cryoinfarcted tissue strips (n=5). Showing engraftment and start of differentiation, both the skeletal (26-kDa) and the cardiac (30-kDa) TnI isoforms were detected 5 days after operation in treated ventricular muscle strips. Interestingly, as early as 2 weeks after transplantation, an almost complete switch to the adult cardiac TnI isoform was observed, suggesting accelerated differentiation of the transplanted cardiomyocytes (Figure 4a). These experiments show that the transplanted cells are physiologically intact and differentiate faster.

**Contractility and Heart Function**

To investigate the functional contribution of contractile proteins, maximal force development at 30 μmol/L Ca^{2+} was determined in permeabilized muscle strips. In the tissue strips harvested from the lesioned areas without engrafted cells, almost no contractile force development (36.9±27 μN, n=5, Figure 4b) was measured. In clear contrast, a significant increase of force development (221.4±52 μN, n=5, Figure 4b) was observed when strong EGFP fluorescence confirmed engraftment of a large number of cells. When the active isometric force per cross-sectional area of tissue strips containing transplanted cells was normalized, it amounted to ≈10% of controls (Figure 4b, inset). To assess whether this force could be generated by the engrafted cardiomyocytes, their total number was counted in tissue slices yielding ≈2000 (n=4) cells, ≈22% of control strips. With their smaller volume taken into account, the theoretical force per cross-sectional area of tissue strips containing transplanted cardiomyocytes lies in the range of 11% of control, close to our experimentally determined value. When fibroblasts instead of embryonic cardiomyocytes were transplanted, almost no contraction (48.6±16.6 μN, n=3) was detected. To evaluate whether this recovery of contractility correlated with an improvement in vivo, echocardiographic analysis was performed. M-mode images in the region of the cryoinfarct...
tion demonstrated that in sham-operated mice, contractility remained low during 2 weeks of observation (Figure 5a), whereas in animals that received transplants, a clear time-dependent improvement was seen (Figure 5b). For detailed assessment of therapeutic efficacy, wall motion scores were determined in the B mode. When the wall motion scores in the anterolateral region of the left ventricle were compared, consistently bad values were found in sham-operated mice, whereas a significant improvement was seen in mice after cellular cardiomyoplasty over time (Figure 5c). When the left ventricular ejection fraction was determined 4 days after operation, no difference was noted between the untreated (28.4±5.3%, n=5) and the treated (25.2±6.6%, n=5) groups. Two weeks after operation, however, global left ventricular function was significantly improved in the cardio-
omyoplasty group (50.6±4.9%, n=5) versus controls (18.4±4.7%), in which a steady deterioration over time occurred (Figure 5d, P<0.01). These data show a remarkable recovery of left ventricular function after cardiomyoplasty.

Animal Survival

A main goal of this study was to clarify whether cellular cardiomyoplasty has an impact on clinical outcome. The time course of survival showed a significant (log-rank P<0.05, Breslow test P=0.02) improvement in the group with cardiomyoplasty (Figure 6). Fourteen days after operation, mortality in the treated group was reduced by almost 50% compared with the group with cryolesion but without treatment. At later time points, no further difference in survival was found.

Discussion

The present study provides compelling evidence that cellular cardiomyoplasty results in a significant improvement of postoperative survival in a large group of animals. Although other groups have previously provided indirect evidence for improved left ventricular function,2,14 our data demonstrate for the first time that transplanted embryonic cells (1) differentiate, (2) are physiologically intact, (3) partially re-
store contractile force, and (4) improve regional and global left ventricular performance. Prominent differences in mor-
tality between sham-operated mice and mice with cardiomy-
oplasty occur at a time when the cardiomyocytes are not yet differentiated and echocardiography shows no clear func-
tional improvement. We therefore presume that the better clinical outcome is also related to decreased compliance of scar tissue1 and possible beneficial effects of the injected cardiomyocytes on scar formation.15 Nevertheless, because experiments that used fibroblasts instead of embryonic car-
diomyocytes did not yield any recovery of contractile func-
tion, the use of noncontractile cell types for cellular cardio-
myoplasty is questionable, as suggested by other groups.2,16 The formation of intercalated disks and the passage of lucifer yellow from EGFP-positive cells to native cardiomyocytes suggest at least some degree of electrical coupling between the grafted cells and the native myocardium. Future studies should analyze this important topic in detail.

Therapeutic strategies using human embryonic cardiomyo-
cyes for cell replacement will be limited because of ethical and immunological problems. Although grafting of autolo-
gous skeletal muscle tissue is devoid of these problems,17,18 lack of electrical coupling19 and different contractile proper-
ties20 pose considerable limitations to their therapeutic use. Therefore, the use of embryonic stem cells,21 somatic stem cells,22 and/or their in vivo transdifferentiation23 represent potential alternatives in the treatment of cardiovascular dis-
eses, even more so because bone marrow mobilization improves repair and survival of infarcted mice.24 It remains to be proved, however, that embryonic/somatic stem cell-
derived “cardiomyocytes” display typical cardiac-like func-
tional characteristics and augment long-term survival.

Acknowledgments

This project was supported by grants from the Deutsche Forschungs-
gemeinschaft (FI 276/3-1 and 4-1), by BONFOR, and by Koenl Fortune. We thank C. Boettinger for help with the cell culture, Dr R. Meyer for advice in the dissociation, and Dr O. Manzek for help with flow cytomtery. We are grateful to Dr T. Kolbe for breeding the transgenic C57/B16 mice, Dr W. Oswald for assistance in the mouse colony, and Dr O. Wiestler for helpful comments on an earlier version of the manuscript.

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Circulation. 2002;105:2435-2441; originally published online April 29, 2002; doi: 10.1161/01.CIR.0000016063.66513.BB
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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